Allergology International (1998) **47**: 205–212

Original Article

Mouse bone marrow-derived mast cells acquire responsiveness to substance P after co-culture with 3T3 fibroblasts in the presence of stem cell factor

Eishin Morita, Takaaki Hiragun, Toshihiko Tanaka, Yoshikazu Kameyoshi, Tsutomu Okabe, Michihiro Hide and Shoso Yamamoto

Department of Dermatology, Hiroshima University School of Medicine, Hiroshima, Japan

Abstract

Connective tissue-type mast cells degranulate in response to a neurogenic peptide, substance P, whereas bone marrow-derived mast cells (BMMC) do not respond to this stimulant when prepared with a combination of interleukin-3 and interleukin-4. In the present study we demonstrated that BMMC obtained from three different strains of mice, NC, BALB/c and C57BL/6, which are immature mast cells low in histamine content and unresponsive to substance P, increased 10 to 100-fold in their histamine content and acquired responsiveness to substance P after co-culture with NIH/3T3 fibroblasts in the presence of 10 ng/mL of stem cell factor (SCF). This change was observed after 1 week of co-culture and increased over a 3 week period, whereas 3T3 fibroblasts or SCF (100 ng/mL) alone was insufficient to duplicate this phenotypic change in BMMC. It is suggested that the response to substance P of mast cells is not through NK-1 receptors but rather through a different mechanism, since the reverse transcriptase-mediated polymerase chain reaction technique failed to show expression of NK-1 receptor mRNA in BMMC after co-culture as well as before co-culture.

Key words: co-culture, mast cells, stem cell factor, substance P, 3T3 fibroblasts.

Correspondence: Eishin Morita, Department of Dermatology, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan.
E-mail: emorita@ipc.hiroshima-u.ac.jp

Received 8 December 1997. Accepted for publication 20 April 1998.

Introduction

Mast cells originate from hematopoietic cells located in bone marrow. Precursors of mast cells circulate in the blood, reach tissues, and then differentiate depending on the micro-environment of the tissue. Mouse mast cells that reside in serosal cavities and skin, connective tissue-type mast cells (CTMC), differ in a number of ways with respect to phenotype from mouse mast cells that reside in mucosa of the intestine, mucosal mast cells (MMC). The CTMC are stained with safranin and berberine sulfate, have heparin in their granules, and have a relatively high histamine content (approximately 10 pg/cell), whereas the MMC do not react to these dyes, have predominantly chondroitin sulfates in their granules, and have a low histamine content (approximately 1 pg/cell).[1,2] In addition, CTMC degranulate in response to neurogenic peptides such as substance P, vasoactive intestinal peptide, and somatostatin, from 10^{-6} to 10^{-4} mol/L, whereas MMC respond weakly or hardly at all to these peptides.[3]

By culturing bone marrow cells *in vitro* with interleukin (IL)-3 and IL-4, mast cells can be easily obtained in a pure preparation (BMMC). These cells cause degranulation by stimulation with IgE and antigen. They are thus thought to be useful for investigating the biochemical events involved in degranulation. These cells resemble MMC but are still immature. They also differ from CTMC histochemically and functionally. One remarkable difference in function between BMMC and CTMC is responsiveness to neurogenic peptides. Bone marrow-derived mast cells fail to react to neurogenic peptides, such as substance P, whereas CTMC release significant amounts of histamine in response to 10^{-5} mol/L substance P.[3] The mechanism of this functional differentiation in mast cells is unclear.

Several studies demonstrated that when the BMMC are co-cultured with mouse skin-derived 3T3 fibroblasts, the BMMC acquire biochemical characteristics resembling CTMC; that is, the mast cells acquire the ability to be stained with safranin, to increase histamine content, and to synthesize heparin.[4,5] Fibroblasts thus play a key role in the differentiation of mast cell precursors into CTMC. Also of interest is the fact that a potent mast cell growth factor, stem cell factor (SCF), has been identified in fibroblasts, and is encoded in the Sl locus and ligand for the c-kit proto-oncogene product.[6] Numerous studies both *in vitro* and *in vivo* have shown the importance of SCF in the development of mast cells.[7]

In this study we demonstrated that mouse BMMC acquire responsiveness to substance P by co-culture with NIH/3T3 fibroblasts in the presence of soluble SCF, and that this response of co-cultured mast cells is not due to expression of a specific substance P receptor, NK-1.

MATERIALS AND METHODS

Animals

Six-week-old BALB/c and C57BL/6 male mice were purchased from Nihon SLC (Hamamatsu, Japan). NC mice were provided by Dr J Hayakawa, Institute for Experimental Animals, School of Medicine, Kanazawa University, Japan.

Growth factors and chemicals

Mouse recombinant IL-3, mouse recombinant IL-4, and mouse recombinant SCF were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Substance P and human serum albumin (HSA)-conjugated dinitrophenol (DNP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Rat anti-DNP-IgE was purchased from BioSource International (Camarillo, CA, USA).

Cell culture

NIH/3T3 fibroblast cell line was obtained from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan) and cultured in α–minimal essential medium (αMEM) (Dainippon) supplemented with 5% calf-serum, 2 mmol/L glutamine, 100 IU/mL penicillin G and 100 μg/mL streptomycin. Bone marrow-derived mast cells were obtained by culturing bone marrow cells from 6-week-old male mice as previously described.[8] Briefly, bone marrow cells were suspended at a density of 1×10^6 cells/mL in αMEM supplemented with 10% fetal calf-serum (FCS), 50 μmol/L 2-mercaptoethanol, 2 mmol/L glutamine, 100 IU/mL penicillin G, 100 μg/mL streptomycin, 5 ng/mL IL-3, and 2 ng/mL IL-4. The cell suspension was then transferred into a 25 cm^2 culture flask and cultured for over 4 weeks by replacing half of the medium with fresh medium each week. After 4 weeks, more than 98% of nonadherent cells were stained positively with alcian blue.

Co-culture of BMMC and 3T3 fibroblasts

In this study 3T3 fibroblasts were suspended in 0.5 mL of αMEM supplemented with 10% FCS, 2 mmol/L glutamine, 100 IU/mL penicillin G, 100 μg/mL streptomycin (complete αMEM) at a density of 6×10^4 cells/mL. A total of 0.5 mL of suspension was seeded in each well of the four-well multidishes (Nunclon Delta SI, Roskilde, Denmark) and cultured until confluence was attained. The BMMC suspension (3×10^4 cells) in complete αMEM were seeded on the NIH/3T3 fibroblast monolayers and cultured in either the presence or absence of growth factors. Half of the medium was replaced every 48 h during the culture. The number of mast cells in co-culture after resuspension in αMEM by trypsinization was calculated using the following formula: number of mast cells/dish $= A \times C/B$, where A is the total number of cells in a culture dish, B the total number of cells in a cytocentrifuge specimen, and C the number of alcian blue-positive cells in the same specimen.

Alcian blue/safranin staining of cultured cells

Cells suspended in 70 μL of αMEM with 1% bovine serum albumin were cytocentrifuged on glass slides, air dried, and fixed in Carnoy's solution (30% chloroform, 10% acetic acid in ethanol) for 20 min. The cells fixed on glass slides were incubated with 0.5% alcian blue in 0.3% acetic acid for 60 min, washed with water, and further incubated with 0.1% safranin in 1% acetic acid for 5 min. After being washed again with water, the cells were embedded in a mounting reagent. Connective tissue-type mast cells from the peritonea of normal mice were used as positive controls for safranin staining.

Histamine release assay

Suspensions of BMMC were equilibrated at 37°C for 5 min, and then incubated in 1.5 mL polypropylene test tubes containing serially diluted substance P in complete αMEM supplemented with 5 ng/mL IL-3 and 2 ng/mL

IL-4 for 20 min. Substance P was dissolved in saline containing 0.04% acetic acid at a concentration of 10^{-3} mol/L and stored at –80°C prior to use. Ion composition of αMEM was Ca^{2+} 1.8 mmol/L, K^{+} 5.3 mmol/L, Mg^{2+} 0.8 mmol/L, Na^{+} 141 mmol/L, Cl^{-} 125 mmol/L, SO_4^{2-} 0.8 mmol/L, CO_3^{2-} 24 mmol/L and PO_4^{3+} 1 mmol/L. The final volume (0.5 mL) in each tube contained 2×10^5 cells. After centrifugation (4°C, 150*g*, 5 min), supernatants were mixed with the same volume of 0.5 N perchloric acid, vortexed, and centrifuged at 12 000*g* for 10 min to precipitate the protein. Cell pellets in the test tubes were resolved in 1 mL of 0.25 N perchloric acid to release histamine.

Co-cultured cells were fed by 0.45 mL of fresh medium and equilibrated at 37°C for 5 min. Either a one-tenth volume (0.05 mL) of serially diluted substance P solution or medium alone was added to the co-culture, which was further incubated for 20 min. Supernatants of the co-cultures were transferred into 1.5 mL polypropylene test tubes and centrifuged (4°C, 150*g*, 5 min) to precipitate cell components. The supernatants were mixed with the same volume of 0.5 N perchloric acid to precipitate the protein. Both the cell pellets in the test tubes and the co-cultured cells were resolved in 1 mL of 0.25 N perchloric acid to release residual histamine.

In all experiments, histamine release was monitored by stimulation with DNP and anti-DNP IgE to establish that the mast cells were responsive to stimulation. Briefly, mast cells were incubated with 0.5 μg/mL anti-DNP IgE overnight and washed twice with medium before a series of concentrations of HSA-DNP was added. After 30 min incubation at 37°C, histamine content was determined in the supernatant and the cell pellets.

Histamine content in samples was determined by using an automated histamine analyzer (Tosoh Corporation, Osaka, Japan). Histamine release was expressed as a percentage of the total cellular content of the histamine as calculated by the formula:

$$\text{histamine in supernatant}/(\text{histamine in pellet} + \text{histamine in supernatant}) \times 100$$

Reverse transcriptase-mediated polymerase chain reaction (RT-PCR)

Polyadenylated RNA was directly isolated from cells or from brain tissue by using a Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA, USA). cDNA was synthesized from 0.1 μg of polyadenylated mRNA using a cDNA Cycle Kit (Invitrogen) and analyzed by using a polymerase chain reaction (PCR) as described previously.[9] The primers used were: mouse *c-kit* sense primer; 5′-GTCAGATGGA-CTTTCAAGACC-3′ (nucleotide position 218–238); mouse *c-kit* antisense primer; 5′-CACTCCAGAATCGTCAACTCT-3′ (nucleotide position 878–898);[10] mouse NK-1 receptor sense primer; 5′-AGGACAGTGA-CCAATTATTTCCTGG-3′ (nucleotide position 4–28); mouse NK-1 receptor antisense primer; 5′-CTGCTGGATGAACTTCTTAAGGTAG-3′ (nucleotide position 645–669).[11] The reaction conditions were denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min over 35 cycles in a DNA thermal cycler (Astec PC700, Fukuoka, Japan). The PCR products (10 μL) were electrophoresed in 1.5% agar gels in 1 × TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 2 mmol/L Na_3 EDTA) at a constant 100 V for 2 h. Products were visualized by ethidium bromide over a UV transilluminator (CSF-20B, CosmoBio, Tokyo, Japan) and photographed. Predicted sizes of PCR products were 681 bp for *c-kit* and 666 bp for NK-1 receptor.

Statistical analysis

Data were analyzed using the Mann–Whitney *U*-test. Results were expressed as either the mean ± SD or mean ± SEM. Probability (*P*) values less than 0.05 were considered to be significant.

RESULTS

Co-culture of BMMC with 3T3 fibroblasts

By culturing bone marrow cells with a combination of 5 ng/mL IL-3 and 2 ng/mL IL-4 for 4 weeks, a virtually pure mast cell preparation (BMMC) was available in three different strains: NC, BALB/c, and C57BL/6. Histamine content of these BMMC varied as indicated in Table 1.

When 3×10^4 BMMC were co-cultured with mouse 3T3 fibroblasts in complete αMEM, only approximately 50% of the initially seeded BMMC were still present after 1 week, 30% after 2 weeks, and 10% after 3 weeks. In contrast to this progressive loss of mast cells, when co-cultured with mouse 3T3 fibroblasts in the presence of SCF the growth of BMMC was enhanced in a dose-dependent manner, and the growth reached a plateau above 10 ng/mL of SCF (data not shown). With 10 ng/mL of SCF, the number of mast cells more than doubled in the first week, but thereafter decreased gradually. After 3 weeks of co-culture in the presence of 10 ng/mL SCF, the

Table 1. Staining and histamine content of mast cells

Mouse type	BMMC* Safranin (%)		Histamine (pg/cell)	Co-cultured mast cells** Safranin (%)		Histamine (pg/cell)
NC	0 ± 0***	0.67 ± 0.35	(*n* = 14)	31 ± 5	7.4 ± 4.7	(*n* = 6)
BALB/c	0 ± 0	0.04 ± 0.04	(*n* = 6)	10 ± 3	4.3 ± 1.6	(*n* = 4)
C57BL/6	0 ± 0	0.09 ± 0.12	(*n* = 8)	7 ± 2	3.3 ± 2.0	(*n* = 4)

*Bone marrow-derived mast cells (BMMC) were obtained by culturing bone marrow cells in the presence of 5 ng/mL IL-3 and 2 ng/mL IL-4 for 4 to 8 weeks.

**Co-cultured mast cells were obtained by culturing BMMC further with NIH/3T3 fibroblasts in the presence of 10 ng/mL stem cell factor (SCF) for 3 weeks.

***Values are mean ± SD.

number of mast cells per well was $5.0 \pm 1.5 \times 10^4$ in NC, $4.0 \pm 2.3 \times 10^4$ in BALB/c, and $3.8 \pm 1.1 \times 10^4$ in C57BL/6 ($n = 4$, mean ± SD in each case). Compared with the initial value, the amount of histamine per cell increased significantly during co-culture in the presence of 10 ng/mL SCF (Table 1). After 3 weeks, the histamine content of co-cultured mast cells was enhanced considerably more than that of BMMC kept in suspension culture with IL-3 and IL-4. Bone marrow-derived mast cells were not stained by safranin at all, whereas 10–30% of the mast cells became safranin-positive when co-cultured with 3T3 fibroblasts in the presence of 10 ng/mL SCF (Table 1).

Substance P-induced histamine release from mast cells

Bone marrow-derived mast cells did not secrete histamine when exposed to substance P in a range from 10^{-7} to 10^{-5} mol/L among the three different strains of mice (NC, BALB/c and C57BL/6), although these cells released up to 50% of total histamine when stimulated by 10–1000 ng/mL HSA-DNP after being sensitized by anti-DNP IgE antibody. When BMMC were maintained with 100 ng/mL SCF in suspension culture for 3 weeks, no remarkable response was observed to 10^{-7} to 10^{-5} mol/L of substance P (data not shown). Bone marrow-derived mast cells co-cultured with 3T3 fibroblasts in the absence of additional growth factors decreased in number; 3 weeks later only approximately 10% of the mast cells survived. These mast cells still contained low amounts of histamine, but did not respond to substance P. However, they began to respond to substance P 1 week after being placed in co-culture with 3T3 fibroblasts in the presence of 10 ng/mL SCF (Fig. 1). By 3 weeks, co-cultured mast cells released approximately 20% of total histamine when exposed to 10^{-5} mol/L substance P (Fig. 1). Histamine

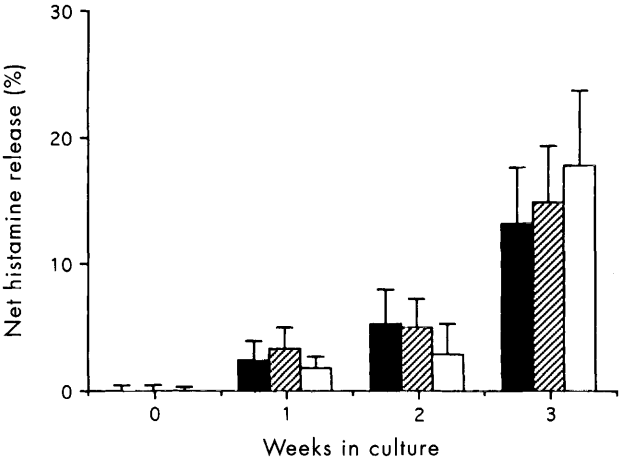

Fig. 1 Histamine release with substance P in co-cultured mast cells. Bone marrow-derived mast cells from three different strains of mice (NC, ■; BALB/c, ▨; and C57BL/6, □) were co-cultured with NIH/3T3 fibroblasts in the presence of 10 ng/mL stem cell factor and incubated with 10^{-5} mol/L substance P for 20 min. Percent spontaneous release in the absence of substance P was subtracted from measured release. Results are from four experiments performed in duplicate and are expressed as mean ± SD.

was released in a dose-dependent manner in a range from 10^{-7} to 10^{-5} mol/L (Fig. 2a). In contrast, BMMC grown by themselves did not show any response to substance P in concentrations up to 10^{-5} mol/L. This unresponsiveness of mast cells was constant during the suspension culture with 5 ng/mL IL-3 and 2 ng/mL IL-4 for up to 6 months (data not shown). When activated with HSA-DNP after sensitization with 0.5 μg/mL IgE, both BMMC and co-cultured mast cells released significant amounts of histamine in similar dose-dependent manners (Fig. 2b). Although percentage histamine release was found to be similar in BMMC and co-cultured mast cells,

the absolute amounts of histamine released were much greater in co-cultured mast cells because the histamine content of co-cultured mast cells was 10 to 100-fold greater than that of BMMC. The results obtained with co-cultured mast cells were similar for each of the three different strains of mice (Fig. 3).

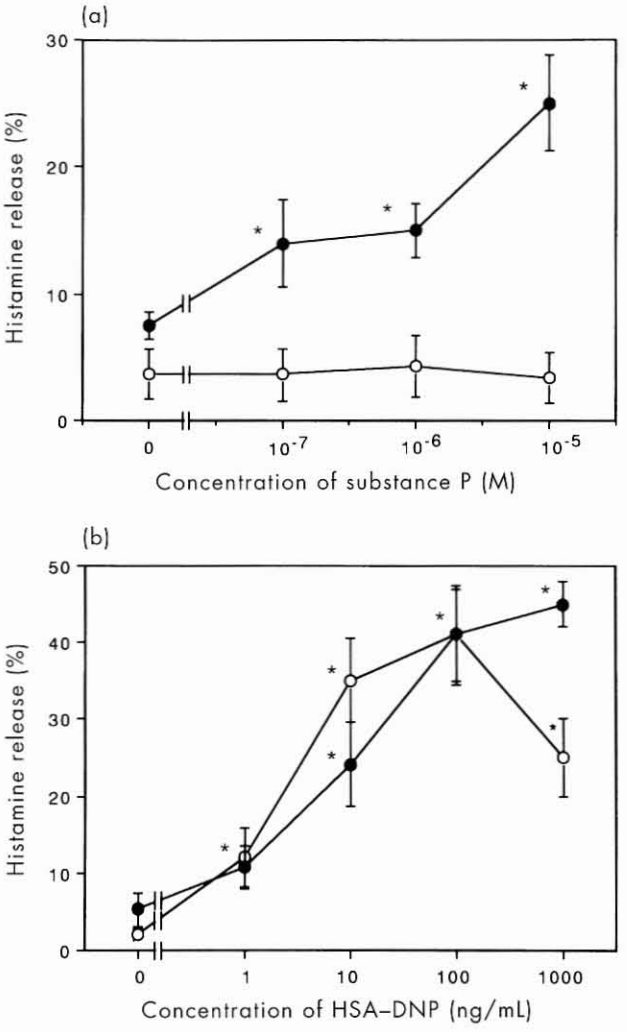

Fig. 2 Effect of culture conditions on histamine release from bone marrow-derived mast cells (BMMC). BMMC from NC (a strain of mouse) were cultured for 3 weeks with 5 ng/mL IL-3 and 2 ng/mL IL-4 (○, suspension) or co-cultured for 3 weeks with NIH/3T3 fibroblasts in the presence of 10 ng/mL stem cell factor (●, co-culture). Mast cells were (a) activated for 20 min with various concentrations of substance P and (b) sensitized with 0.5 μg/mL anti-DNP IgE overnight and activated for 30 min with various concentrations of human serum albumin (HSA)-DNP. Results are from three experiments performed in duplicate and expressed as mean ± SEM. $^*P < 0.05$, compared with spontaneous release.

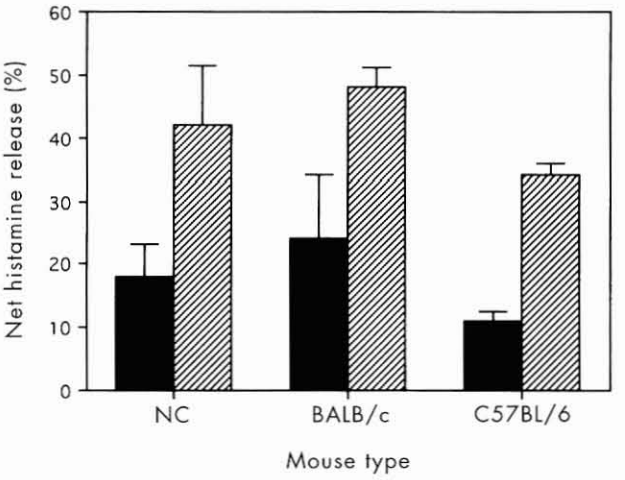

Fig. 3 Histamine release from co-cultured mast cells obtained from three different strains of mice (NC, BALB/c and C57BL/6). Bone marrow derived mast cells (BMMC) were co-cultured for 3 weeks with NIH/3T3 fibroblasts in the presence of 10 ng/mL stem cell factor, and activated for 20 min with 10^{-5} M substance P (■), or activated for 30 min with 100 ng/mL human serum albumin (HSA)-DNP after being sensitized with 0.5 μg/mL anti-DNP IgE overnight (▨). Per cent spontaneous release without stimuli was subtracted from measured release. Results are from four experiments performed in duplicate and expressed as mean ± SD.

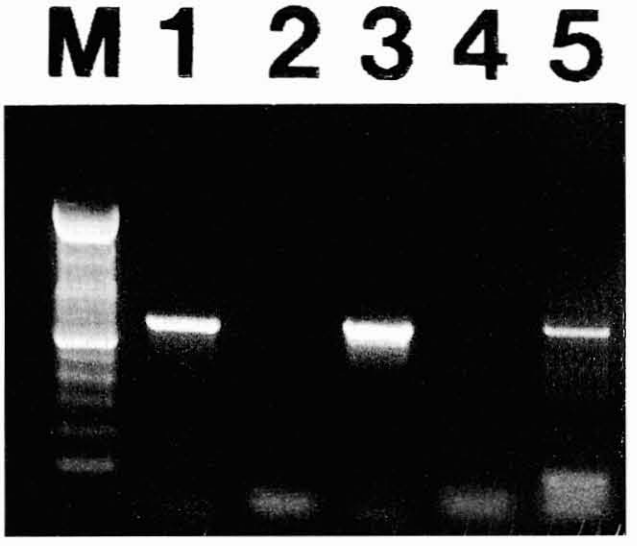

Fig. 4 Expression of NK-1 receptor mRNA. Bone marrow-derived mast cells (BMMC) from NC, before and after co-culturing for 3 weeks with NIH/3T3 fibroblasts in the presence of 10 ng/mL stem cell factor, were tested for NK-1 receptor mRNA after RT-PCR. Lane M, 100 bp DNA ladder; lane 1, *c-kit* in BMMC; lane 2, NK-1 receptor in BMMC; lane 3, *c-kit* in co-cultured mast cells; lane 4, NK-1 receptor in co-cultured mast cells; lane 5, NK-1 receptor in mouse brain. Predicted sizes of PCR products were 681 bp for *c-kit* and 666 bp for NK-1 receptor.

NK-1 receptor mRNA expression

Since the NK-1 receptor recognizes substance P, we examined NK-1 receptor mRNA expression in BMMC and co-cultured mast cells by the RT-PCR technique. Analysis of PCR amplification with specific primers for the NK-1 receptor gene failed to show mRNA expression for the NK-1 receptor in either BMMC or co-cultured mast cells in the presence of 10 ng/mL SCF (Fig. 4).

Discussion

Substance P is a member of the tachykinin family and resides in primary sensory cutaneous neurones, both in dorsal root ganglia and in distal nerve terminals. Antidromic stimulation causes substance P to be released from nerve terminals, resulting in a wheal and flare reaction. In this reaction, histamine released from skin mast cells by stimulation with substance P is considered to play a crucial role, because pretreatment with H-1 antagonists markedly reduces the magnitude of the reaction induced by substance P,[12,13] and substance P induces histamine release in rat peritoneal mast cells and in human cutaneous mast cells.[3,14,15]

Bone marrow-derived mast cells differ from CTMC functionally in that they are unresponsive to neurogenic peptides. Our results indicate that fibroblasts play a key role in the functional differentiation of BMMC into CTMC. When BMMC were co-cultured with 3T3 fibroblasts in the presence of 10 ng/mL SCF, BMMC increased in both number and histamine content per cell. One week later these co-cultured mast cells gained responsiveness to substance P, and this response was strengthened during co-culture (Fig. 1). It is unlikely that this functional phenotypic change was due to stimulation by SCF alone, because BMMC maintained with up to 100 ng/mL soluble SCF exhibited no response to substance P. Bone marrow-derived mast cells co-cultured with 3T3 fibroblasts in the absence of additional growth factors still contained low amounts of histamine, but did not respond to substance P. Although fibroblasts are a known source of SCF, the amount of SCF expressed on NIH/3T3 fibroblasts appears to be insufficient for a phenotypic change of mast cells. These results suggest that a combination of a sufficient amount of SCF with some other factor from fibroblasts is responsible for the phenotypic change of BMMC into CTMC.

We learned of studies in which BMMC acquired responsiveness to compound 48/80 or substance P after co-culture with Swiss 3T3 fibroblasts in combination with WEHI-3 conditioned medium[16] or a high amount of SCF.[17] In addition, Ogasawara *et al.* demonstrated experimentally that a labile soluble substance other than SCF is involved in the mast cell/fibroblast interaction.[17] The mechanism underlying this phenotypic change still remains to be determined.

The mechanism for substance P-induced degranulation in mast cells is also obscure. Substance P binds preferentially to a G-protein-coupled neurokinin-1 (NK-1) receptor and is involved in various biological functions, such as pain transmission and smooth muscle contraction.[18,19] In a mast cell line CFTL 12, which responds to substance P (10^{-9} to 10^{-7} mol/L) to release tumor necrosis factor-α, substance P receptor mRNA is constitutively detected by Northern blotting.[20] However, the existence of NK receptors on mast cells has been questioned.[14,21,22] The effective concentrations of substance P to trigger histamine release in CTMC (10^{-7} to 10^{-5} mol/L) are well above those required to stimulate neurokinin receptors (10^{-9} mol/L).[14,15] In addition, examination using Arg-Pro-Lys-Pro$(CH_2)_{11}CH_3$ has revealed that the N-terminal basic amino acid residues (Arg, Lys) are essential for histamine releasing activity and that interaction between the C-terminal sequence of substance P and mast cells is non-specific and dependent only upon the hydrophobic nature of that part of the peptide,[23] while C-terminal tachykinin consensus residues (Phe-X-Gly-Leu-Met-NH_3) are essential for binding to NK receptors,[24] suggesting uninvolvement of the NK-1 receptor in substance P-induced mast cell activation. This was supported by our results, which indicated that concentrations of substance P to induce histamine release in co-cultured mast cells were comparable to those needed to activate CTMC, and that NK-1 receptor mRNA was not detected in either BMMC or co-cultured mast cells upon application of the RT-PCR technique. In substance P-induced mast cell activation a direct activation model of G-proteins has been proposed.[21,22,25] Hydrophobic C-terminal residues of substance P are considered to penetrate the membrane to activate membrane-coupled G-proteins in a receptor-independent manner, resulting in histamine release. However, the effective dose of substance P to activate G-proteins (10^{-5} to 10^{-3} mol/L) is much higher than that required for mast cell activation.[25] The fact that neuraminidase treatment induces the desensitization of mast cells to substance P suggests that negative charges of sialic acid residues facilitate the action of substance P. Another substance P receptor with low affinity, although not yet identified, might be involved in

the activation of CTMC. Mast cell activation by substance P is not likely to be mediated through the NK-2 receptor, which shows 10 to 100-fold lower affinity for substance P than neurokinin A, because neurokinin A, a high affinity-agonist to the NK-2 receptor, does not stimulate mast cell degranulation. Nor have we detected NK-2 receptor mRNA in cocultured mast cells or BMMC by RT-PCR (data not shown). A difference in Ca^{++} concentration in the reaction solution was also unlikely to have affected the responsiveness of co-cultured BMMC, because the Ca^{2+} concentration in the reaction was kept constant in the medium, somewhat below physiological condition throughout the experiments.

In this study we have achieved a functional differentiation of BMMC into cells strongly resembling CTMC by co-culture with NIH/3T3 fibroblasts in the presence of lower concentration of SCF, presumably resembling the micro-environment of dermis in skin. Our results imply that this functional phenotypic change of mast cells is seen in general for mice, because BMMC obtained from three different strains of mice (NC, BALB/c and C57BL/6) acquired responsiveness to substance P after co-culture with 3T3 fibroblasts in the presence of SCF. Our results also imply that the response to substance P of co-cultured BMMC is not through the NK-1 receptor.

REFERENCES

1 Enerbäck L. Mast cell heterogeneity: the evolution of the concept of a specific mucosal mast cell. In: Befus AD, Bienenstock J, Denburg LA, eds. *Mast Cell Differentiation and Heterogeneity.* New York: Raven Press, 1986; 1–26.

2 Miller HRP, Huntley JF, Newlands GFJ *et al.* Mast cell granule proteases in mouse and rat: A guide to mast cell heterogeneity and activation in the gastrointestinal tract. In: Galli SJ, Austen KF, eds. *Mast Cell and Basophil Differentiation and Function in Health and Disease.* New York: Raven Press, 1989; 81–91.

3 Shanahan F, Denburg JA, Fox J, Bienenstock J, Befus D. Mast cell heterogeneity: Effects of neuroenteric peptides on histamine release. *J. Immunol.* 1985; **135**: 1331–7.

4 Dayton EL, Pharr P, Ogawa M *et al.* 3T3: fibroblasts induce cloned interleukin 3-dependent mouse mast cells to resemble connective tissue mast cells in granular constituency. *Proc. Natl Acad. Sci. USA* 1988; **85**: 569–72.

5 Levi-Schaffer F, Dayton EL, Austen KF *et al.* Mouse bone marrow-derived mast cells cocultured with fibroblasts: Morphology and stimulation-induced release of histamine, leukotriene B_4, leukotriene C_4, and prostaglandin D_2. *J. Immunol.* 1987; **139**: 3431–41.

6 Zebo KM, Williams DA, Geissler EN *et al.* Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for c-*kit* tyrosine kinase receptor. *Cell* 1990; **63**: 213–24.

7 Lantz CS, Huff TF. Differential responsiveness of purified mouse c-*kit*+ mast cells and their progenitors to IL-3 and stem cell factor. *J. Immunol.* 1995; **155**: 4024–9.

8 Nakamura K, Tanaka T, Morita E, Kameyoshi Y, Yamamoto S. Enhancement of fibroblast-dependent mast cell growth in mice by a conditioned medium of keratinocyte-derived squamous cell carcinoma cells. *Arch. Dermatol. Res.* 1994; **287**: 91–6.

9 Morita E, Lee D-G, Sugiyama M, Yamamoto S. Expression of c-*kit* ligand in human keratinocytes. *Arch. Dermatol. Res.* 1994; **286**: 273–7.

10 Qiu F, Ray P, Brown K *et al.* Primary structure of c-*kit*: relationship with the CSF-1/PDGF receptor kinase family—oncogenic activation of v-*kit* involves deletion of extracellular domain and C terminus. *EMBO J.* 1988; **7**: 1003–11.

11 Cook A, Elliott D, Ahmed M *et al.* Molecular evidence that granuloma T lymphocytes in murine *Schistosomiasis mansoni* express an authentic substance P (NK-1) receptor. *J. Immunol.* **1994**: 152: 1830–5.

12 Hagermark O, Hokfelt T, Pernow B. Flare and itch induced by substance P in human skin. *J. Invest. Dermatol.* 1978; **71**: 233–5.

13 Jorizzo JL, Coutts AA, Eady RAJ, Greaves MW. Vascular responses of human skin to injection of substance P and mechanism of action. *Eur. J. Pharmacol.* 1983; **87**: 67–76.

14 Shibata H, Mio M, Tasaka K. Analysis of the mechanism of histamine release induced by substance P. *Biochem. Biophys. Acta* 1985; **846**: 1–7.

15 Ebertz JM, Hirshman CA, Kettelkamp NS, Uno H, Hanifin JM. Substance P-induced histamine release in human cutaneous mast cells. *J. Invest. Dermatol.* 1987; **88**: 682–5.

16 Sakaguchi N, Saito H, Iikura Y. The stimuli releasing histamine from murine bone marrow-derived mast cells (BMMC): 3. Effect of coculture with 3T3 fibroblasts on the histamine releasability of BMMC. *Arerugi.* 1992; **41**: 519–25.

17 Ogasawara T, Murakami M, Suzuki-Nishimura T, Uchida MK, Kudo I. Mouse bone marrow-derived mast cells undergo exocytosis, prostanoid generation, and cytokine expression in response to G protein-activating polybasic compounds after coculture with fibroblasts in the presence of c-*kit* ligand. *J. Immunol.* 1997; **158**: 393–404.

18 Fong TM, Yu H, Huang RRC, Strader CD. The extracellular domain of the neurokinin-1 receptor is required for high affinity binding of peptides. *Biochem.* 1992; **31**: 11806-11.

19 Regoli D, Dion S, Rhaleb N-E *et al.* Selective agonist for receptors of substance P and related neurokinins. *Biopolymers* 1989; **28**: 81–90.

20 Ansel JC, Brown JR, Payan DG, Brown MA. Substance P selectively activates TNF-α gene expression in murine mast cells. *J. Immunol.* 1993; **150**: 4478–85.

21 Repke H, Bienert M. Mast cell activation—a receptor-independent model of substance P action? *FEBS Lett.* 1987; **221**: 236–40.

22 Aridor M, Traub LM, Sagi-Eisenberg R. Exocytosis in Mast cells by Basic secretagogue: Evidence for direct

activation of GTP-binding proteins. *J. Cell Biol.* 1990; **111**: 909–17.

23 Repke H, Piotrowski W, Bienert M, Foreman JC. Histamine release induced by Arg-Pro-Lys-Pro $(CH_2)_{11}CH_3$ from rat peritoneal mast cells. *J. Pharm. Exp. Ther.* 1987; **243**: 317–21.

24 Watling KJ, Krause JE. The rising sun shines on substance P and related peptides. *Trends Pharmacol. Sci.* 1993; **14**: 81–4.

25 Mousli M, Bronner C, Landry Y, Bockaert J, Rouot B. Direct activation of GTP-binding regulatory proteins (G-proteins) by substance P and compound 48/80. *FEBS Lett.* 1990; **259**: 260–2.